10. Study Design, Methodology and Data Analysis

The study will aim to recruit volunteers who are between 18 and 50 years. It is anticipated 60 volunteers will be required. The study will be conducted at the CMR research facility King's College HospitalVolunteers will be from the UK Armed Forces or local to King's College Hospital (KCH). We are particularly interested in volunteers with a history of fainting or presyncope although this is not a strict inclusion criteria.

Volunteers will be asked not to drink alcohol the night before the test and until all testing has been completed (approximately 9-10 days). Volunteers will not be required to stay overnight at the Research Facility.

Participants will be briefed either collectively or individually prior to attending KCH. A copy of the PIS will be provided. Recruitment will be performed by email or phone from interested parties. A 24 hour 'cooling off' period will be employed following any briefings. Before briefing units and recruiting UK Armed Forces personnel permission will be sought from the individuals chain of command ensure there is no clash with respect to unit duties.

Upon arrival at KCH a medical screening questionnaire will be conducted with the view to identifying subjects with exclusion criteria (section 14b). We will attempt to provide this questionnaire to participants prior to attendance at the KCH to decrease the chance of a participant making a wasted trip. Following obtaining informed consent the subject will be double-blind randomised to one of 3 groups:

Placebo/Drink A (Group A): Glucose
Drink B (Group B): Sodium Chloride, Glucose
Drink C (Group C): Sodium Chloride, Glycerol

The sodium chloride dose will be split into 2x 90mmol doses for drink A and drink B. All doses and osmolality calculations (by dead reckoning and freezing point depression) are available on request. MOD Intellectual Property have requested the authors to limit promulgation of exact dosing in case the effect size is substantial enough for warranting patent application. The drink components are entirely formulated from commercially available 'off-the-shelf' products at concentrations which, one would not anticipate, would result in sustained side-effects. A head-to-head assessment of the components of each drink is not the purpose of this study, rather the effect of each drink on orthostatic tolerance.

Day 1

Tests will again be conducted in the mornings with test volunteers asked to have only a light breakfast avoiding caffeine and the avoidance of strenuous exercise for at least 12 hours prior to testing. Women will be asked to make a note of the date of their last period (we would prefer to schedule testing on a day when they do not have, or expect, their period because this may influence orthostatic tolerance).

- **Urine Measures**

Urinary sodium (24 hour), urine osmolality, urinary specific gravity, urinary colour and urinary volume (24 hour) will be measured. Following completion of the days testing a 24 hour urine collection bottle will be given to the volunteers. They will be instructed to exclusively urinate in this bottle and to return it the following day when they will attend for further testing.

- **Orthostatic tolerance**

Volunteers will be asked to undergo a “tilt test” to assess cardiovascular reflex control and orthostatic tolerance (measured as time to presyncope, or near fainting, in minutes). This technique has been shown previously to be reproducible, reliable, and to have high sensitivity and specificity for differentiating persons with differing orthostatic tolerance, or for examining the effects of interventions aimed at improving orthostatic tolerance[2,12,15,64–70]. This involves the following:

a. A standard 3 lead electrocardiogram (ECG) will be recorded to assess heart rate and rhythm.
b. Beat-to-beat blood pressure will be determined using the Finometer blood pressure monitoring device. This consists of a small Velcro cuff placed around the middle finger that pulses gently against the digital arteries and records and displays blood pressure with every heartbeat.
c. We will measure the cardiac stroke volume by echocardiography. These measures will be taken intermittently at various times during the test. Volunteers can can choose whether to be bare chested, or whether to wear a hospital gown to cover their chest.
d. We will place a strap over the participants’ knees and a box over their legs that seals against their waist (a bit like a canoe skirt). The strap is to help them stand in a relaxed position without minimal muscle activity in the legs. If participants contract their leg muscles it may increase blood pressure and so improve orthostatic tolerance. For the same reason, we will ask participants to try not to move their legs too much during testing (some movement is inevitable). The box is placed over their legs so that we can apply lower body negative pressure to the legs later on in the test without disturbing the monitoring.

Once the monitors are in place we will make recordings from them for 15 minutes of supine rest. We will then tilt the table into an upright position (at 60 degrees). We will make recordings from the monitors throughout the upright portion of the test. After 20 minutes of standing, while still upright, we will apply lower body negative pressure to the box over their legs. This typically feels a little draughty on the legs however, it is not painful or unpleasant. The effect mimics prolonged standing by inducing further hydrostatic/gravitational stress on the lower limbs and enables the precise determination of orthostatic tolerance. Without the addition of lower body negative pressure, most healthy volunteers can tolerate orthostatic stress for extremely protracted time periods that become barriers to testing. The addition of the lower body negative pressure permits the precise determination of orthostatic tolerance within a maximum of 50 minutes. We will apply the lower body negative pressure at three different levels for 10 minutes each (-20mmHg, -40mmHg and -60mmHg).

The test will be stopped and the participant returned to the supine position immediately if:

- They complete the whole procedure (30 minutes lying down, 20 minutes standing, and 30 minutes of lower body negative pressure).
- They experience symptoms of dizziness or lightheadedness (presyncope) and/or blood pressure or heart rate begin to decrease. If the blood pressure declines to a systolic pressure of 80mmHg or less, sometimes associated with symptoms of presyncope such as lightheadedness, the test will be immediately terminated. The beat-to-beat determination of blood pressure ensures prompt termination of the test as the blood pressure begins to decline, and avoids undue hypotension. This ensures that volunteers typically experience only modest hypotension for a few seconds, and are only mildly symptomatic (if at all). In some individuals presyncope is associated with slowing of the heart rate. For this reason, new onset bradycardia of less than 50bpm while upright will be taken as criteria to terminate the test. The tilt table is returned to supine with removal of the negative pressure within seconds. This enables prompt resolution of orthostatic hypotension or bradycardia, and so

minimises the discomfort to the participant, and ensures actual fainting is very unlikely.

- The participant requests for the test to stop.

If the volunteer experienced symptoms or signs of presyncope at the end of the test, lying down will quickly resolve them. The monitors will be removed and any residue from the ultrasound gel will be removed.

Participants who exhibit an orthostatic tolerance greater than their age predicted average[17] (detailed below) will then be excluded.

Orthostatic tolerance testing of normal subjects(min)						
	Males			**Females**		
Age (years)	20-35	36-50	>50	20-35	36-50	>50
Mean (SD)	35±11.1	35±13.5	35±13.5	29±11.9	31±14.3	37±18.3

We will thank the participants for taking part at this point and pay 25% of the honorarium (see Section 18).

Reasons for excluding volunteers at this point:

Evidence would suggest that volunteers will experience a 20% increase in orthostatic tolerance following salt loading[28] with a hypothesised further 20% increase with the addition of creatine monohydrate and glycerol. Individuals whose orthostatic tolerance exceeds 35 minutes therefore are likely to 'top out' on the test with the potential to confound results. Individuals with a more than average orthostatic tolerance are significantly less likely to faint, if subjected to typical physiological stresses, so are unlikely to benefit from oral plasma volume expansion for the purposes of this outcome measure.

Whilst we are interested in recruiting participants whom have previously fainted, we will not make this a mandatory requirement as some individuals who have never fainted, have a poor orthostatic tolerance, largely due to effective compensatory mechanisms eg postural sway.[71]

We are attempting to improve our predictive capabilities regarding orthostatic tolerance with a study currently being performed. If this work on risk index yields results we will relay this information in briefings (Section 15- recruitment) to inform personnel of characteristics, which they will be able to self-identify with, eg height, weight, race, sex, history of syncope/ anxiety etc.

Day 2

Tests will usually be conducted in the mornings. On the day of the test volunteers will be asked to have only a light breakfast avoiding caffeine, and should avoid strenuous exercise for at least 12 hours prior to testing.

- **Physiological examination:**

Height, weight, body composition measures (via bioelectrical impedance), blood pressure measures

- **Ultrasound derived measures of haemodynamic status:**

These are non-invasive cardiovascular measurements of vascular and cardiac function including echocardiography and pulse wave Doppler arterial studies. Participants will be asked to remove any clothing on their upper body. They can choose to complete the testing bare chested, or to change, in privacy, into a hospital gown.

(see King's College London Department of Clinical Pharmacology Standard Operating Procedure- Appendix 6)

- **Blood measures:**

Baseline blood and urine biochemical measures as well as a urinalysis will be performed. These will include, salt-sensitivity analysis, serum osmolality, renin, aldosterone, haematocrit, haemoglobin, Plasma volume will be estimated using the Dill and Costill method[62]. Due to potential concerns that a prior positive COVID-19 infection may affect orthostatic tolerance due to autonomic dysfunction[72] we will also check COVID-19 serology once.

Intervention phase (Day 3 to Day 9):

The volunteer will be given 14 pouches of powder and 14 small bottles of liquid. The powder will be made up of one of the following ingredients: Drink A, Drink B or Drink C as detailed above. The small bottle of liquid will contain glycerol for Group B only. For Group A-C and placebo this small bottle will contain water. Volunteers will also receive a 1L water bottle.

Volunteers will be instructed to put the pouch of powder and the small bottle of liquid in the water bottle and add water to fill the water bottle to 1L. Participants will be instructed to do this first thing in the morning with a further at lunchtime. Volunteers will be instructed to do this every day. Volunteers will be asked to drink the drink as soon as comfortably possible after mixing. Volunteers will be instructed that if they experience side effects and you wish to stop taking the supplement we would ask that they contact the chief investigator or the Research Facility at King's College London.

We have opted not to specifically control the individual's diet. The reasons for this are as follows: the salt dose is sufficient to counteract salt loads in the individual's diet. We would envisage the main confounder would be alcohol and additional supplement exposure, which we have asked subjects to abstain from. Participants who are taking diuretic medication or receiving blood or blood products for medical conditions will be excluded.

Day 10
The testing performed on Day 2 will be repeated.

Day 11
The orthostatic tolerance test (Day 1) will be repeated.

Processed samples of blood and urine may be frozen and stored at St Thomas' Hospital. All the samples will be labelled with a number. They will be anonymised. Measurements will be performed in batches. The samples will be destroyed within two years. Unused samples will be disposed of through approved procedures for clinical waste. St Thomas' has a HTA licence for the storage of human tissue.

In the unlikely event the study results indicate the existence of a previously undiagnosed medical condition, then the Chief Investigator will ensure that the volunteer is provided with appropriate information and, as necessary, counselling. Clinical information will be summarised in a letter which will be given to the volunteer and addressed to their responsible medical practitioner. A copy will be provided to the participant, and where the findings may have significant ramifications, for the purposes of safeguarding, a copy will also be sent the participant's medical officer (Appendix 4). The volunteers will be required to consent to this prior to participation in the study. In the unlikely event that the participant withdraws their consent for potentially threatening clinical information to be shared with their medical officer, the Chief Investigator, with the support of physician co-investigators, will use clinical

judgement. This is foreseeably limited to abnormalities in the focussed echocardiography, blood or urine work. In all cases volunteers will have the opportunity to discuss the findings with a civilian consultant physician (Dr Luca Faconti).

A sealed copy of the letter will be kept in the participant's study file.